Optimization of Simple DNA Extraction Method Suitable for Diverse Microorganisms

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ABSTRACT

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To date no simple DNA extraction method was reported to be efficient and adapted to various organisms and microorganisms. Moreover, this approach is hard, time consuming and rely on the use of liquid nitrogen. In order to obtain highly purified nucleic acids free of contaminants that could interfere with the amplification reaction during PCR, adequate and easy extraction methods should be developed. During this work, an efficient, fast and economical method for the isolation of high-quality DNA from fungi, bacteria and viruses is described. Those DNA extractions were performed without the use of liquid nitrogen. Besides, the protocol used allowed obtaining very good DNA concentrations that can be utilized at 1/50, regardless the origin of the analyzed samples, whether freshly collected, conserved in calcium chloride, or frozen at -80°C for long time (more than 10 years). The quantity and the quality of the extracted DNA by this method are enough high to perform cloning, PCR simplex or multiplex and also other DNA manipulation techniques.

Keywords: DNA, extraction, liquid nitrogen, microorganisms, PCR

Development of PCR-based molecular techniques has become the principal method to detect and characterize pathogens, and to understand the principle factors of molecular evolution (Mishra et al. 2008). The first step in molecular analysis is the preparation of purified, high molecular weight DNA and looking for an adequate method for DNA isolation leading to the development of various protocols.

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Reported DNA isolation methods such as sodium dodecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB) are limited to certain organisms (Ahmed et al. 2009; Margam et al. 2010), and protocols must to be adjusted to each type of tissue because of the presence of secondary metabolites (polysaccharides, polyphenols, alkaloids and flavanoids) accumulated by plants or insect tissues, bacterial or fungal material which causes damage to DNA and/or inhibit Taq polymerases and restriction endonucleases (Calderón-Cortés et al. 2010; Sahu et al. 2012). Those approaches also rely on long-lasting period (through the need for overnight incubation) and hardworking, and some methods are expensive and/or ineffective (Milligan 1998).

Furthermore, DNA must be purified from cellular material mainly to avoid degradation, the main trouble encountered during DNA extraction (Weishing et al. 1995; Sahu et al. 2012). For this reason, in most cases an initial grinding stage with liquid nitrogen is performed (Rogers and Bendich 1994) to break down cell wall material and allow access to DNA (Tan and Yiap 2009), although liquid nitrogen has many disadvantages related to its availability, cost, transportation, storage and quick evaporation especially in Tunisia where temperatures are relatively high. Hence, in order to simplify classical DNA isolation methods, commercial genomic DNA extraction kits have been developed (Jobes et al. 1995; Cheng et al. 2003). However, these methods are usually either expensive or not readily available, require large amount of cellular material (in grams) to be grounded and are not suitable for a large number of samples (Wang et al. 2011).

A method requiring neither the use of commercial expensive kit nor liquid nitrogen and mainly adapted to divers organisms and microorganisms (plants, insects, fungi and bacteria) need to be developed for DNA isolation.

The aim of this study is the optimization of a protocol based on buffer method described by Carling 2004 without need of liquid nitrogen, and available for different plant tissues and microorganisms. We describe in this paper one consistent protocol to extract DNA from diversified cell origins. The optimized protocol is characterized by the use of high salt concentrations (2M NaCl) to remove polysaccharides (Fang et al. 1992), polyvinyl pyrrolidone (2% PVP) to remove polyphenols, and phenol-chloroform-isoamyl alcohol for extraction and finally DNA precipitation by cold isopropanol and extended RNase treatment.

This method is relatively simple, quick, low cost and suitable for an open laboratory environment, a method that avoids the use of liquid nitrogen and needs only small amount of sample, fresh buffer and mortar.

The results have proved that this protocol is valid for DNA extraction from different plant and insect tissues, bacteria, and fungi and the extracted DNA could be directly used in experiments, such as PCR, enzyme digestion, etc.

MATERIALS AND METHODS

DNA extraction

DNA was extracted from cultured microorganisms (fungi, bacteria) and from virus infected plant organs. A quantity of 50 mg of fresh or conserved samples (ie mycelium, bacterial cell, animal or vegetal tissues) was mixed with 500 µl of freshly prepared and preheated at 65°C grinding buffer composed of 5 ml of extraction buffer (0.35 M Sorbitol, 0.1 M Tris pH 7.5, 5 mM EDTA), 5 ml of lysis buffer (0.2 Tris pH 7.5, 0.05 M EDTA, 2 M NaCl, 2% CTAB), 2 ml of sodium lauryl sarkosyl (NLS at 5% w/v), 0.05 M de sodium bisulfate and 2% w/v of polyvinylpyrrolidone (PVP) 25000-30000). Then, the samples were grinded in mortar by adding sterilized quartz sand. Samples were immediately vortexed and incubated at 65°C for 40 min with agitation every 5 min. After adding an equal volume of phenol chloroform-isoamyl alcohol (24/24/1), tubes were homogenized by gentle tube inverting 30 times and centrifuged at 13200 rpm during 5 min; the supernatant was then recovered in Eppendorf tubes containing 80 µl of sodium acetate 3 M. Precipitation was performed by adding 0.8 volume of isopropanol and mixing gently by inverting the tube 20 times. After incubation during at least 1 h at -80°C, tubes were centrifuged for 5 min at 13200

rpm at 4°C. Precipitated nucleic acids collected were washed twice with 500 µl of ice-cold 70% ethanol and centrifuged for 5 min at 13200 rpm. The pellets were air dried and resuspended in 50 µl bi distilled (DEPC) treated water (0.1% diethylpyrocarbonate) containing 0.5 µg of RNAse, then incubated at 37°C for 30 min. Purified DNA samples may be stored for short time at -20°C prior to analysis or for long time at -80°C until use.

Qualitative and quantitative analysis of extracted DNA.

For quality and yield assessments, electrophoresis was done for all kind of samples of DNA extracts, in 0.8% agarose gel, stained with ethidium bromide. Visualization was performed by gel documentation system and DNA quantification was assessed by using 1-kbp DNA ladder (Fermentas). DNA purity was evaluated by calculating the absorbance ratio DO260/280. Good-quality DNA will have an A260/A280 ratio of 1.7-2.0.

DNA concentration was calculated using the relationship that an A260 of 1.0 = 50 µg/ml pure DNA. Thus, concentration in µg/µl = A260 reading × dilution factor × 50µg/1000.

DNA yield (µg) = DNA concentration × total sample volume (µl)

PCR analysis.

Viral DNA amplification. Total DNA extracted using the described method from different plant species (tomato, watermelon, faba bean, pepper and some weeds) showing *Tomato yellow leaf curl virus* (TYLCV) symptoms freshly collected or frozen at -80°C for long time (more than 10 years) was subjected to amplification with two specific primer pairs designed by Davino et al. 2008 to target the IR region. (TY2222 [5' GTCGTTGGCTGTCTGTTGTC-3'], TY 255 [5'-GGTTCGTAGGTTTCTTCAAC TAG-3'], TY2463 [5'-GGTTCGTAGGT TTCTTCAACTAG-3'], TY247 [5'-TGG TTCCCCATTCTCGTGG-3']).

Amplification reaction was done in final volume of 20 µl containing 1x PCR reaction buffer (Pol Jena Bioscience), 0.2 mM of each primer, 0.2 mM of each dNTPs, 2 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Pol Jena, Bioscience). PCR was performed in an Applied Biosystem Thermocycler programmed as follows: initial denaturation at 94°C for 4 min followed by 34 cycles (denaturing at 94°C for 30 s, annealing at 60°C for 90s and extension at 72°C for 90s) and a final extension cycle at 72°C for 10 min.

The second 2 primer pairs (TY209, TY575, TY613, TY1363) amplifying a portion of the CP and the V2 gene of TYLCV was described by Pellegrin et al. 2008. Amplification reactions were done in 20 µl contained 2x Master Mix (Quiagen, France), 0.2 µM of each primer and 1 µl of total DNA (diluted 1/20 to 1/50 depending on the initial concentration of the sample) per reaction mixture. PCR was performed in an Applied Biosystem Thermocycler with an initial denaturation at 95°C for 15 min followed by 34 cycles (denaturing at 94°C for 30 s, annealing at 63°C for 90 s and extension at 72°C for 90 s), and a final extension at 72ºC for 7 min).

Fungal DNA amplification. The ITS regions and 5.8rDNA were amplified for *Athelia rolfsii*, *Macrophomina phaseolina* and *Fusarium* spp. isolates using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') developed by White et al. 1990. Amplification reactions (50 µl) contained 1x PCR buffer (Promega, USA), 200 µM of each dNTP (Promega, USA), 1.5 mM $MgCl_2$, 1.0 µM of each primer, 1.0 U of Green GoTaq DNA Polymerase (Promega,

USA) and 10 ng of fungus DNA per reaction mixture. PCR was performed in a Biometra Thermocycler programmed with an initial denaturation at 94°C for 5 min followed by 35 cycles (denaturation at 94°C for 60 s, annealing at 50°C for 90 s and extension at 72°C for 90 s), and terminated with a final extension at 72ºC for 7 min.

Bacterial DNA amplification. 16S rDNA region of bacterial DNA was amplified using the fd1 (5-GGAGAG TTAGATCTTGGCTC-3') and rd1 (5-AA G-GAGGTGATCCAGCCGCA-3') primer pair (Weisburg et al. 1991). PCR reactions were carried out in 50 µl reaction mixture containing 1 x PCR buffer (Quiagen) (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 200 µM of each dATP (Quiagen), 1.0 µM of each primer fd1 and rd1, 2 µl of DNA, and 0.5 µl of crude recombinant Taq DNA polymerase (Quiagen). PCR reactions were conducted in a Biometra thermocycler programmed with an initial denaturation at 94°C for 5 min; 31 cycles of denaturation at 94°C for 30 s, annealing at 65 °C for 60 s and extension at 72 °C for 90 s followed by a final extension at 72 °C for 5 min.

Visualization of PCR products.

PCR products were separated on 0.8 and 1.5% agarose gels, respectively for DNA extract or amplified product of more than 1200 bp and for PCR amplified fragments of about 800 bp, stained using ethidium bromide and visualized under UV light.

Sequencing.

The PCR products obtained after amplification of viral, fungal, and bacterial DNA were further purified with a MinElute TMgel extraction kit (Quiagen) or PCR preps DNA purification systems (Promega, USA) according to manufacturer's instructions. Sequencing was performed by Genewiz (England).

Sequences analysis.

Sequences were checked and assembled by the CAP program (http://pbil.univ-lyon1.fr/cap3.php). The BLAST program (http: //blast.ncbi.nlm. nih. gov/Blast.cgi) was used to search for sequences similarities into the DNA databases and sequences were aligned using Geneious software then were deposited in GenBank.

RESULTS

Quantification and qualification of DNA extracted.

After electrophoresis of DNA extracted from different tissues and pathogens, the visualization of agarose gel under UV showed clean pattern of DNA extracted without liquid nitrogen in terms of quantity and quality (Figs. 1, 2). All obtained DNA extracts were very concentrated, and must be diluted 1 to 50 or more before use excepted the DNA purified from *Athelia rolfsii*. The concentration of the extract was relatively low due to the exopolysaccharides that bind to the DNA making it mucilaginous. The problem was resolved by adding one step of purification using ethyl ether before precipitation (Punja and Sun 2001).

The determination of absorbance of nucleic acids confirmed the high quality and quantity of DNA extracted from all tested materials even for sample stored for long time (Fig. 1, Table 1). The ratio 260/280 is ranged from 1.71 to 2.05.

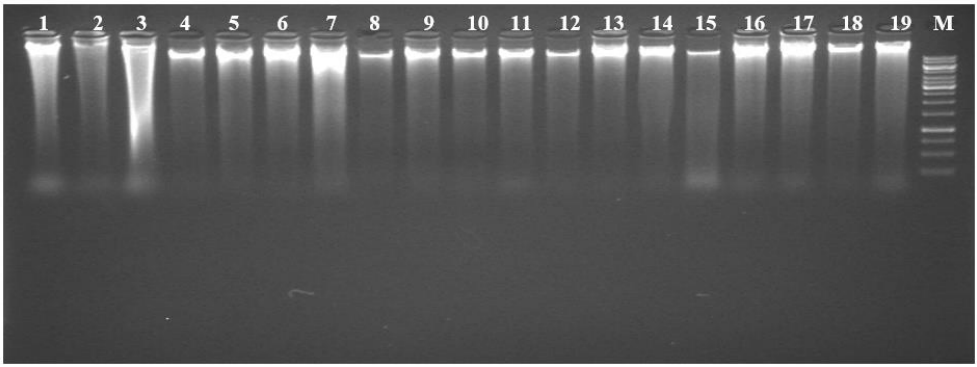

Fig. 1. Agarose gel electrophoresis (0.8%) of 2 µl of extracted genomic DNA purified from various plant tissues freshly collected or frozen: M: 1 Kb DNA Ladder (GeneRuler) Line 2 and 3: extracted DNA from pepper; Line 1, 4, 6, 7, 18 and 19: extracted DNA from tomato freshly collected; Line 5 and 16: extracted DNA from tomato conserved at -80°C since 2009; Line 8 and 9: extracted DNA from watermelon conserved at -80°C since 2005; Line 12 and 13: extracted DNA from faba bean ; Line 10, 11, 14, 15 and 17: extracted DNA from weeds.

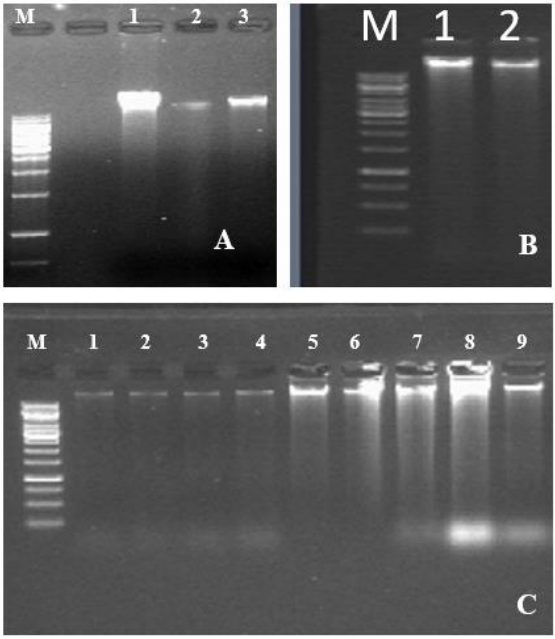

Fig. 2. Agarose gel electrophoresis (0.8%) of 2 µl extracted genomic DNA purified from various tissues and cells; **A**: DNA purified from *Mayetiola destructor*; **B**: DNA purified from Aphids; **C:** DNA purified from *Athelia rolfsii* (Lane 1-4) and *Macrophomina phaseolina* (Lane 5-9).

Table 1. Quantification and qualification of DNA extracted by assessment of optical density

Samples	Do 260	DO 260/280	Concentration (µg/µl)
Fungus sample DNA extracts	0.091-0.539	1.77 to 1.9	0.182*-1.078
Bacteria sample DNA extracts	0.255-0.312	1.71 to1.9	1.02-1.248
Plant tissue sample DNA extract	0.337-0.423	1.79 to 2.05	1.348-1.692
Insect tissue sample DNA extract	0.143-0.182	1.91 to 1.99	0.572-0.728

* Concentration of DNA of mucilaginous extract of *A. rolfsii* was low due to a second step of purification using ethyl ether before precipitation.

To confirm the DNA purity and to test amplification ability extracted DNAs were tested for PCR amplification.

Viral DNA amplification.

Multiplex PCR amplifications of extracted DNA from freshly and or stored vegetable sample for more than 10 years to detect TYLCV species was successfully achieved using primers pair dressed by Davino et al. 2008. Respectively a product of 570 bp for TYLCV and 800 bp for *Tomato yellow leaf curl Sardinia virus* (TYLCSV) were obtained (Fig. 3A). A reproducible PCR product pattern was obtained using different primers (Fig. 3B); there was no sign of degraded DNA after amplification. Moreover, the same extracted DNA was successfully used for a period over 5 years which indicates the reproducibility of the results and the integrity of the DNA.

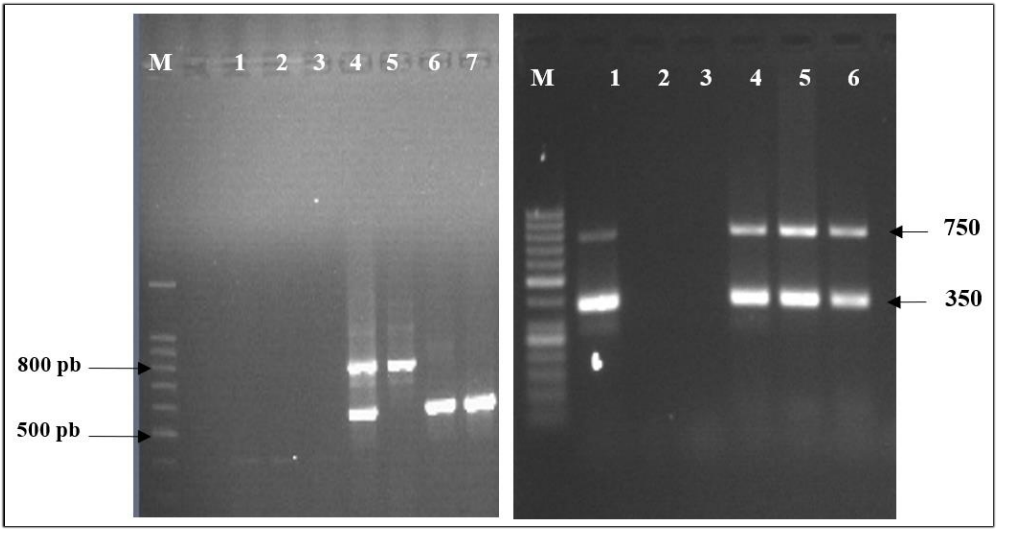

Fig. 3. A: Agarose gel electrophoresis (1.5%) of the PCR products obtained after amplification with the primer pairs TY255, TY2222, TY247 and TY2463. **M**: GeneRuler™50 bp DNA ladder from Fermentas, Lane 1: Negative control, Lane 2, 3, 4, 5, and 6: tomato samples; Lane7: positive control. **B**: Agarose gel electrophoresis (1.5%) of the PCR products obtained after amplification with the primer pairs TY209, TY575, TY613, TY1363: M: GeneRuler 100 pb+ DNA ladder. Lane 1: positive control; Lane2: Negative control, Lane 3, 4, 5, and 6: tomato samples.

Fungal DNA amplification (ITS).

The amplification of ITS region of fungal species gave a major single band of 580 to 600 bp corresponding to four different *Fusarium* species characterized by variable ITS region length (Fig. 4).

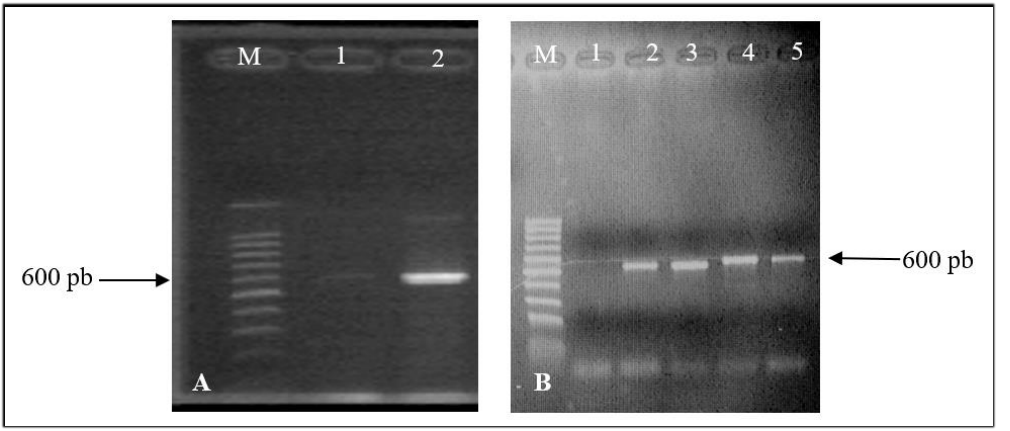

Fig. 4: Amplification of the ITS1-5.8S-ITS2 region for molecular identification. **A:** *Athelia rolfsii*: M: 100 pb molecular marker (invitrogen); Lane 1: negative control; Lane 2: *Athelia rolfsii*; **B:** M: 100 pb molecular marker (invitrogen); Lane 2-5: *Fusarium* spp. The amplification of 16S region of bacterial isolates gave a major single band of 1600 bp (Fig. 5).

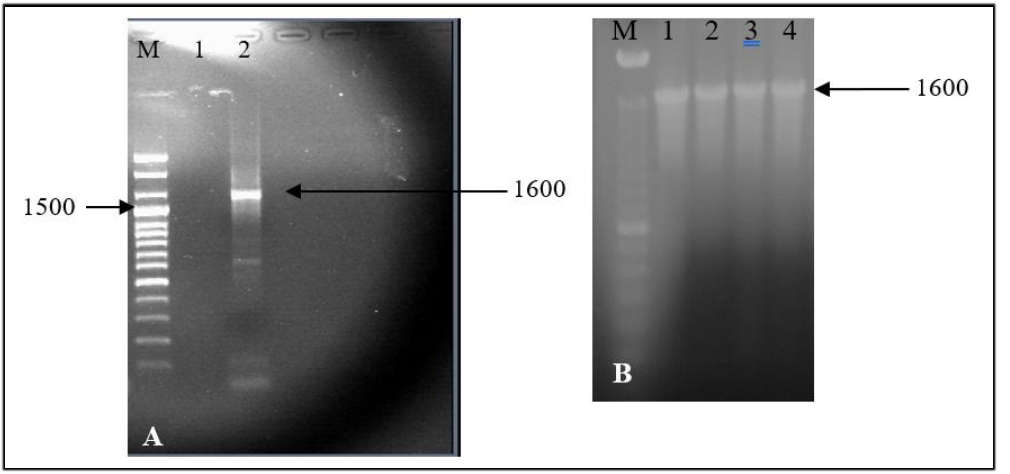

Fig. 5. Agarose gel electrophoresis (1%) of the PCR products obtained after amplification of the 16S rDNA region. **A**: M: Gene Ruler 100pb + DNA ladder; Lane 1: negative control; Lane 2: *Athelia rolfsii*; **B**: M: 100 pb molecular marker (invitrogen); Lane 1-4: *Bacillus* spp.

Sequencing.

The obtained sequences from virus infected vegetable were affiliated to *Tomato yellow leaf curl virus*, and *Tomato leaf curl Sardinia virus* (Zammouri et al. 2014).

Sequencing of bacterial 16S rDNA region and fungal ITS1-5.8S-ITS2 region allowed assigning them respectively to Bacillus genus for bacteria and to *Athelia rolfsii* (Kalai-Grami et al. 2013) and *Macrophomina phaseolina* (Hajlaoui et al. 2015) for fungi.

DISCUSSION

Most of all genomic DNA extraction methods from plant species, from bacteria and mostly from fungi or insects are difficult due to high mucilage (Cassago et al. 2002), high concentrations of phenolics and protein content (Mishra et al. 2008; Calderón-Cortés et al. 2010). Consequently, those protocols require the use of liquid nitrogen for tissue grinding (Aljanabi and Martinez 1997) which is difficult to obtain and to maintain in region like Tunisia characterized with very hot summer and moderate winter. Moreover, liquid nitrogen is not always available. The method presented here makes possible to extract and purified high molecular weight DNA from different plant species freshly collected or stored at -80°C for more than 10 years, either from bacteria, insects (Mayetiola and Aphids), or fungi (*Athelia rolfsii* and *Macrophomina phaseolina* and *Fusarium* spp.). All these DNA extractions were done without the use of liquid nitrogen or time-consuming procedures. The protocol used is technically rapid and relevant on a variety of species regardless the complexity of their genomes. The use of sterilized little fine sand is sufficient for grinding a huge variety of tissues. Besides, the efficiency of this method was confirmed with small amount (50 mg) of fresh or up to 10 years stored samples (-80°C).

The obtained total DNA could be amplified by PCR using different primer pairs and satisfactory amplifications were generated. These results were in concordance to Sharma et al. (2008, 2010) that showed that a good quality of DNA can be isolated without the use of liquid nitrogen.

Moreover, we used the same DNA extracted for PCR amplifications over a period of 5 years and we obtained the same banding pattern which indicates the reproducibility of the results and the integrity of the DNA.

In addition, phenolics, polysaccharides and other secondary compounds accumulated by plants and insect tissues and fungal mycelia induce damage to DNA and/or inhibit restriction endonucleases and Taq polymerases (Calderón-Cortés et al. 2010). In the present study, higher concentrations of cetyltrimethylammonium bromide (2% CTAB) and the addition of antioxidants such as PVP (2%) with lower molecular weight to the extraction buffer further improved the quality of the extracted DNA by removing all phenolics from DNA preparations (Calderón-Cortés et al. 2010). The high quality of obtained DNA could also be attributed to the use of a higher concentration of NaCl. A number of researchers have recommended the use of PVP to face the problem of phenolics (Zidani et al. 2005). The addition of sodium acetate coupled with isopropanol help to purify and precipitate nucleic acid by removing cellular and histone proteins bounded to the DNA (Zidani et al. 2005).

Here we have described a simple, reliable, and cost-efficient DNA extraction method that provides high-quality DNA from fungi, bacteria, insects and plants containing high concentrations of

polysaccharides and polyphenolic compounds. This method allowed also reducing DNA degradation, and the contaminations of extracted DNA and minimize the risk to obtain low yield of DNA due to binding with starches and polysaccharides.

The quantity and the quality of the DNA extracted by this method are high enough to perform amplification using DNA dilution 1/50 and to make a hundred of PCR-based reactions and also to be used in other DNA manipulation techniques such as restriction digestion.

However, this protocol should be improved to limit as much as possible the use of toxic organic substances.

RESUME

Zammouri S., Kalai-Grami L. et Mnari-Hattab M. 2018. Optimisation d'une méthode d'extraction d'ADN simple et valable pour divers microorganismes. Tunisian Journal of Plant Protection 13 (2): 145-155.

Actuellement aucune méthode simple d'extraction efficace et adaptée n'a été rapportée pour isoler l'ADN à partir de divers organismes et microorganismes. En plus, la plupart des protocoles décrits sont assez difficiles, longs et ayant recours à l'utilisation de l'azote liquide. Afin, d'obtenir des acides nucléiques hautement purifiés exempts de contaminants qui pourraient gêner la réaction d'amplification au cours de la PCR, des méthodes d'extraction adéquates et faciles devraient être mises au point. Au cours de ce travail, nous décrivons une méthode efficace, rapide et économique pour purifier les acides nucléiques totaux à partir de champignons, bactéries et virus et ce, sans avoir recours à l'azote liquide. Cette méthode nous a également permis d'obtenir de très bonnes concentrations d'ADN que nous avons utilisé au 1/50 et ce quel que soit l'origine des échantillons analysés, qu'ils soient frais ou séchés au chlorure de calcium ou même conservés à -80°C depuis plus d'une dizaine d'années. La qualité et la pureté des acides nucléiques obtenues permettent leur utilisation pour le clonage, l'amplification par PCR simplex, multiplexe, et diverses autres techniques.

Mots clés: ADN, azote liquide, extraction, microorganismes, PCR

ملخص

الزموري، سامية وليلى قلعي-قرامي ومنية المناري-حطاب. 2018. ضبط طريقة مثلى لاستخراج الحامض النووي DNA بسهولة من كائنات دقيقة مختلفة.

Tunisian Journal of Plant Protection 13 (2): 145-155.

لم يتم إلى حد الآن تسجيل طريقة بسيطة، ناجعة ومتأقلمة مع استخراج الحامض النووي DNA من أنسجة مختلفة. علاوة على ذلك، إن غالبية البروتوكولات المتداولة، إما أن تكون صعبة أو أن تطبيقها يستغرق وقتا طويلا. كما أنها تعتمد على النيتروجين السائل. لذلك، استوجب ضبط طريقة مناسبة وفعالة للحصول على أحماض نووية نقية من كل الشوائب التي يمكن أن تعطل عملية تفاعل البوليميراز التسلسلي PCR. لقد تم من خلال هذا العمل ضبط طريقة سهلة وسريعة وغير مكلفة لتطهير الأحماض النووية الكاملة لعدة عينات من فيروسات وبكتيريات وفطريات، وهذا دون اللجوء إلى استعمال النيتروجين السائل. مكنتنا هذه طريقة من الحصول على أحماض نووية نقية وجيدة التركيز بغض النظر عن نوعية العينة أو طريقة تخزينها (مجففة على باستعمال $CaCl_2$ أو محفوظة منذ أكثر من 10 سنوات في درجة حرارة 80-°س). تم استعمال هذه الأحماض بعد تخفيفها إلى حد 50/1 وكانت النتائج إيجابية وجيدة. مكنتنا النوعية الممتازة من الأحماض النووية التي تحصلنا عليها من استعمالها في الاستنساخ وتضخيم PCR البسيط والمتعدد وكذلك تقنيات مختلفة أخرى.

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